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14. ABSTRACT

The purpose of this study was to determine the role of Notch signaling in lymphatic endothelial cell (LEC) behavior and to determine the effects on tumor vasculature upon Notch inhibition. We hypothesized that inhibiting Notch activity may disrupt tumor (lymph)angiogenesis by changing expression and activity of EC genes. To that end, we have created a treatment agent known as Notch1 decoy (hN1DFc). Activation of Notch changes the gene profile of LEC and changes their in vitro behavior. An orthotopic model of human breast cancer was established. Pilot studies show that expression by these tumors of a mutant VEGF-C induces tumor lymphangiogenesis and lymph node metastasis. These tumors are rich in Notch-positive vasculature, making this a good model for future studies of Notch inhibition on tumor vasculature.

15. SUBJECT TERMS

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Introduction

Tumor size, lymph node involvement, and distant metastases are all important prognostic factors in breast cancer. Interestingly, angiogenesis and lymphangiogenesis are involved in each of these factors. In order for a tumor to grow, invade nearby lymph nodes, and spread to distant parts of the body, it needs blood and lymphatic vessels. The Notch signaling pathway is a cell-fate determining pathway that consists of 4 receptors (Notch 1, 2, 3, 4) and 5 ligands (Delta-like 1, 3, 4 and Jagged 1, 2). When a Notch receptor interacts with a ligand and is activated, a series of proteolytic cleavages release the intracellular domain of the receptor from the cell membrane, allowing it to translocate to the nucleus and act as a transcriptional regulator. It has been established that Notch is present and active in the vasculature. Our lab has demonstrated that Notch can regulate endothelial cell (EC) genes, most notably Vascular Endothelial Growth Factor Receptor 3 (VEGFR-3), whose regulation is mediated by direct binding of Notch in the promoter region. We hypothesize that disrupting Notch activity may interfere with tumor angiogenesis and lymphangiogenesis by disrupting expression and activity of EC genes. To that end, we have created a treatment agent known as Notch1 decoy (hN1DFc).

Body

Task 1. Study how Notch signaling functions in primary lymphatic endothelial cells

Notch function in the behavior of primary blood endothelial cells (BEC) has been established in human umbilical venous endothelial cells (HUVEC). Notch family receptors and ligands (Notch 1, Notch 4, Dll-4, Jag1) are present in HUVEC. Activation of Notch in HUVEC inhibits *in vitro* proliferation, migration, and network formation, presumably by Notch's ability to repress VEGFR-2 (unpublished data).

In order to study Notch function in the behavior of primary lymphatic endothelial cells (LEC) *in vitro*, we established a method of isolating human dermal lymphatic endothelial cells (HDLEC) from neonatal foreskins. Notch family receptors and ligands (Notch 1, Notch2, Notch 4, Dll-4, Jag1) were present in our isolated HDLEC on the transcript **(Figure 1a)** and protein level **(Figure 1b)**.

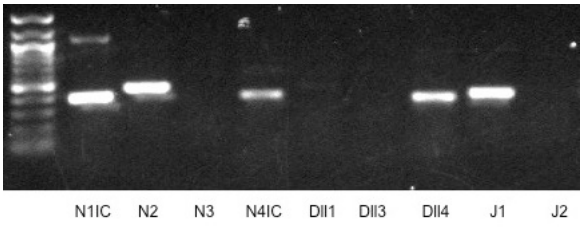

Figure 1a

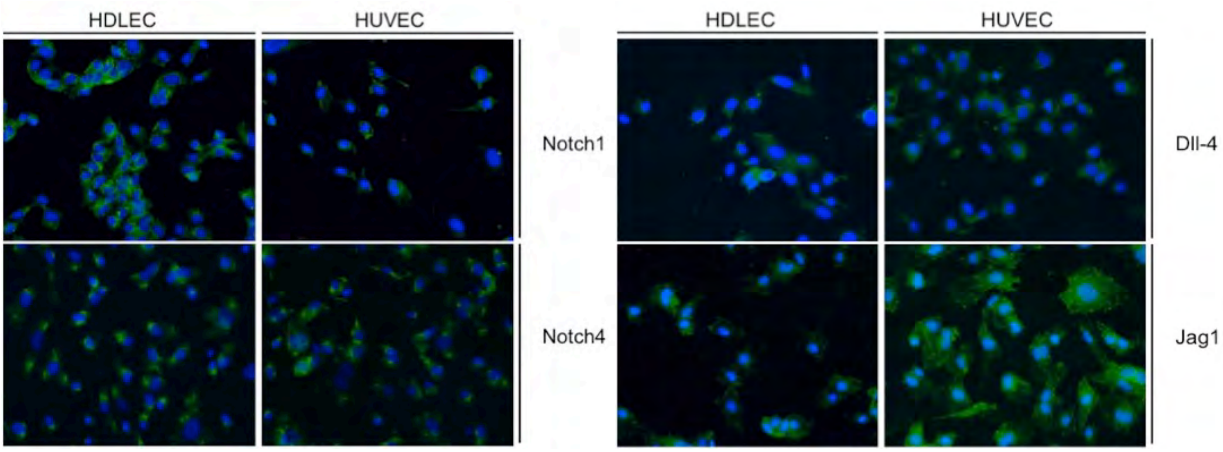

Figure 1b

To activate Notch in HDLEC, lentiviral constructs expressing activated Notch 1 (N1IC) or activated Notch 4 (N4int3) were used. As expected, Notch activation (with either N1IC or N4int3) significantly induced transcripts for direct targets such as Hey1 and Hey2 (data not shown), as well as the LEC gene VEGFR-3 **(Figure 2a)**. Interestingly, Notch activation significantly repressed transcripts for most LEC genes (e.g., Prox1, LYVE1, and podoplanin) **(Figure 2b)**, as well as for VEGFR-2 (a BEC-associated gene, which is also repressed in HUVEC upon Notch activation) (data not shown).

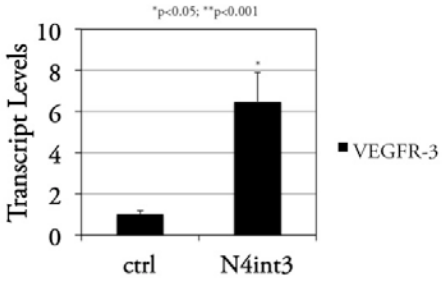

Figure 2a

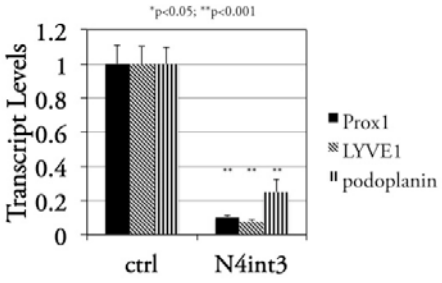

Figure 2b

Effects of Notch activation on HDLEC behavior were tested in various different *in vitro* assays. Briefly, proliferation was tested by seeding equal numbers of cells for all conditions being tested, then quantifying cell number after 4 days of culture. Migration was observed by using a pipet tip to make equal-sized scratches in cell monolayers, then observing the closing of the scratch over a period of 24hrs. Network formation was observed by plating equal numbers of cells in between two collagen gel layers and observing the formation of networks over 4 days of culture. All assays were performed in serum-free endothelial media supplemented with

EGF and VEGF-C. Preliminary data suggest that Notch activation in HDLECs inhibits *in vitro* proliferation, migration, and network formation **(Figure 3a, b, c)**.

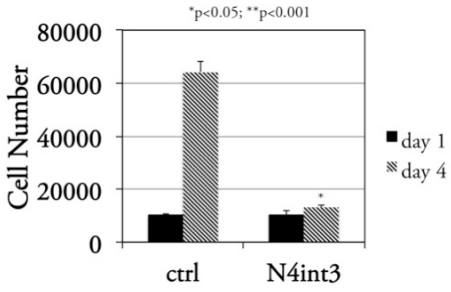

Figure 3a

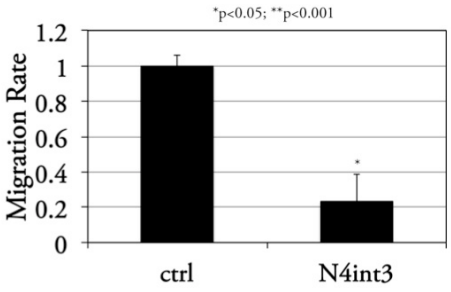

Figure 3b

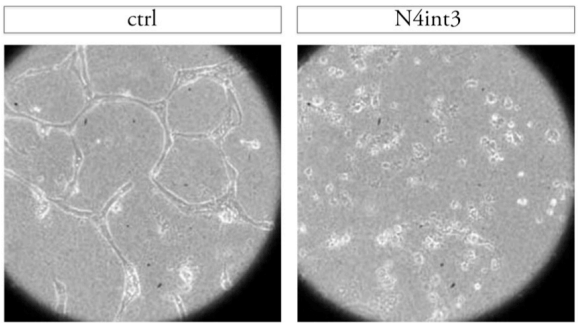

Figure 3c

Tasks 2 and 3. Study Notch function in pathological angiogenesis and lymphangiogenesis

To study the effects of Notch inhibition in breast tumor vasculature *in vivo*, the MDA-MB-231 human breast cancer cell line was used for xenograft studies. An MDA-MB-231 line that stably expresses luciferase (231-luc) was used. Luciferase activity was useful for live imaging of tumor progression throughout the course of tumor studies. A survey revealed that transcripts for both Notch and VEGF receptor family members were present in cultured 231-luc cells (data not shown). Notches 1, 2, and 3; Dll-1 and Dll-4; Jag1; and VEGFR-2 were present. Immunohistochemistry of pilot 231-luc tumors grown in the mammary fat pads of female nude mice demonstrated that Notch family receptors and ligands were indeed expressed by the tumors themselves, as well as in the blood (yellow arrows) and lymphatic (white arrows) vasculature **(Figure 4)**.

The luciferase-expressing cell line was used to generate the following cell lines:

1. GFP
2. VEGF-C_cys156ser (V_c156s)
3. hFc+GFP
4. hFc+V_c156s
5. hN1DFc+GFP
6. hN1DFc+V_c156s

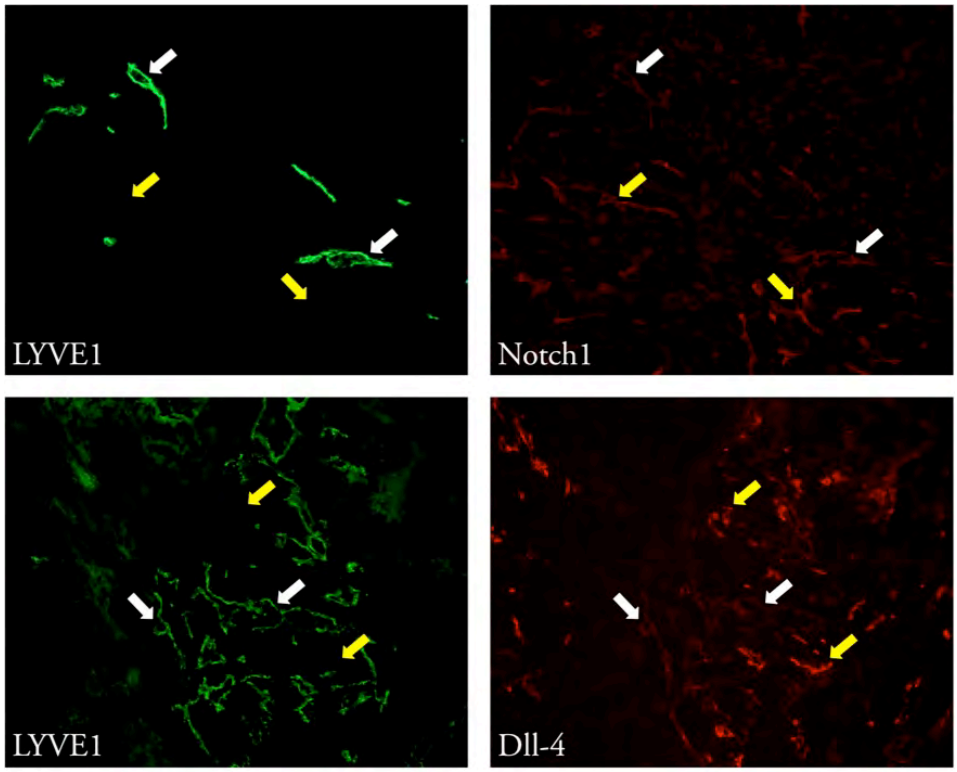

Figure 4

VEGF-C_cys156ser (V_c156s) is a mutant VEGF-C that binds only VEGFR-3. This mutant VEGF-C was used to focus more closely on effects of Notch inhibition on VEGFR-3^{+} vasculature (tumor lymphatics and blood vessels both express VEGFR-3). hN1DFc is Notch 1 decoy (consisting of the 36 EGF-like repeats in the extracellular region of human Notch 1, fused to an Fc tag) that was created in our lab as a pan-Notch inhibitor. Western blot demonstrated that the cell lines expressed and secreted the appropriate proteins (data not shown).

These cell lines were tested to determine whether *in vitro* proliferation, migration, or growth in soft agar was affected. Although 231-luc express Notch receptors and ligands, there were no significant differences in proliferation or migration of the 6 cell lines (data not shown). We were unable to compare colony formation, as the 231-luc cell line does not grow well in soft agar.

Subsequently, pilot tumor studies were performed. In the first pilot study, 231-luc/GFP and 231-luc/V_c156s were used to determine whether V_c156s secretion induces tumor (lymph)angiogenesis and tumor growth/progression. $2*10^{6}$ cells mixed with Matrigel were orthotopically implanted into the right 4^{th} mammary fat pad of 4-6 week old female nude mice. Tumors were allowed to progress for 10 weeks before mice were sacrificed, tumors resected, and lungs as well as lymph nodes were imaged *ex vivo* for metastases. We found

that V_c156s conferred a slight growth advantage over GFP **(Figure 5a)**, and tumor weights at the end of the study were found to be approximately 1.5x greater in V_c156s mice **(Figure 5b)**.

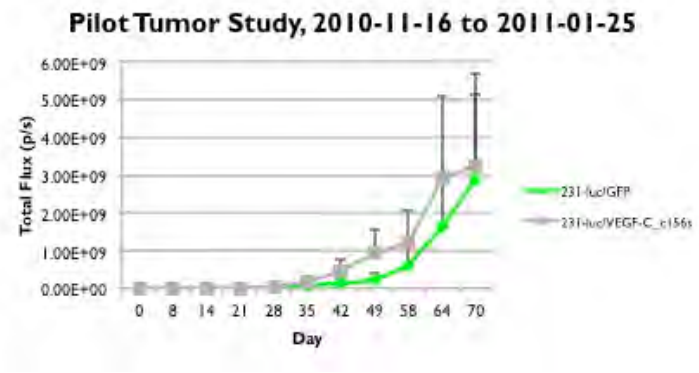

Figure 5a

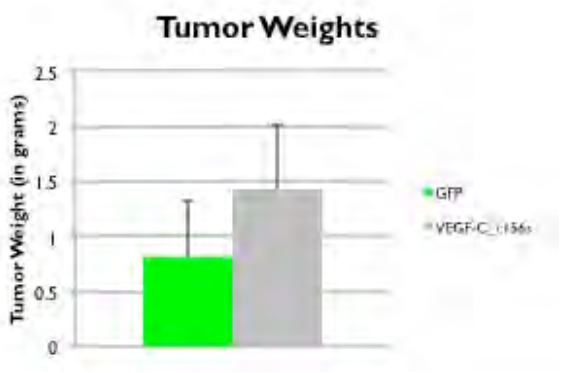

Figure 5b

Ex vivo imaging of lungs and lymph nodes found no difference between groups for lung metastases (33% of mice had lung metastases, data not shown), but a significant difference between groups for metastases to axillary lymph nodes (4 of 9 mice in the V_c156s had axillary lymph node metastases, while 0 of 9 mice in the GFP group had axillary lymph node metastases, data not shown). Quantification of immunostaining for CD31 (a general vessel marker that stains both blood and lymphatic vasculature) revealed that V_c156s tumors had approximately a 1.5-fold increase in $CD31^+$ vasculature, which was not significant (data not shown). Immunostaining for LYVE1 (a lymphatic vessel marker) revealed that V_c156s tumors had an increase in tumor lymphatics, as well as an increase in invasion of lymphatics into the tumor **(Figure 6)**. This correlates with our axillary lymph node *ex vivo* imaging data, suggesting that the increase in tumor lymphangiogenesis in V_c156s tumors may be contributing to increased metastasis to lymph nodes.

Currently, we are working on our second pilot study, in which four lines are being compared: GFP, V_c156s, GFP+hFc, and V_c156s+hFc. The purpose of this pilot study is to show that hFc (which is used as a control for our hN1decoy line) has no effect on tumor vasculature, progression, or growth. Once this is established, we will perform larger-scale tumor studies comparing V_c156s+hFc (control) and V_c156s+hN1DFc to look for effects of Notch inhibition on tumor progression or vasculature.

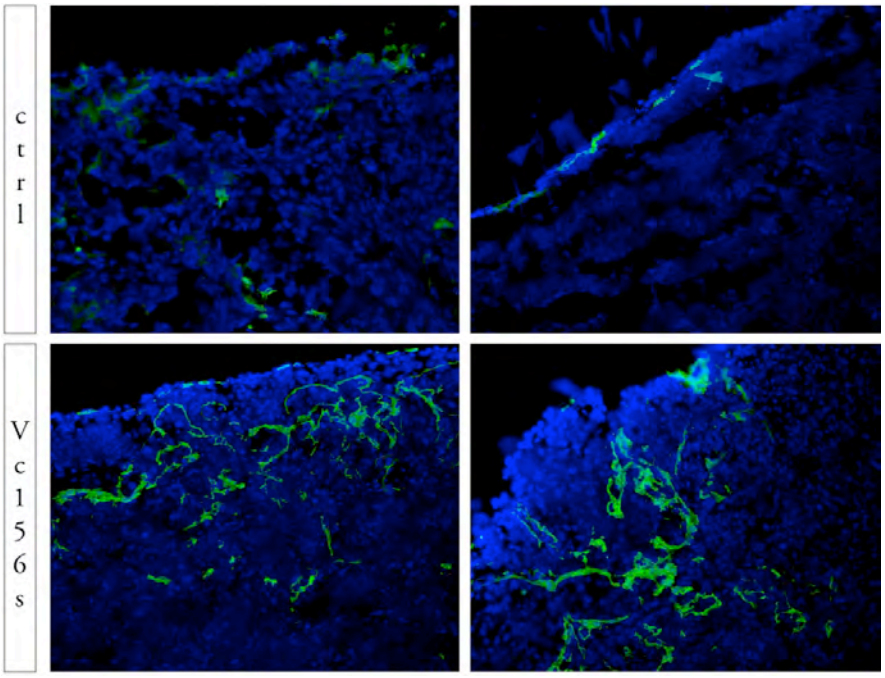

Figure 6

Key Research Accomplishments

- Established a method of isolating primary human dermal lymphatic endothelial cells
- Demonstrated that Notch receptors and ligands are present in lymphatic endothelial cells (LEC)
- Demonstrated that perturbation of Notch signaling in LEC changes the gene profile of LEC
- Demonstrated that perturbation of Notch signaling in LEC inhibits proliferation, migration, and network formation *in vitro*
- Established an orthotopic model of human breast cancer that recruits blood and lymphatic vasculature and metastasizes to lungs and lymph nodes (231-luc). This will be a good model to study how Notch inhibition affects tumor blood and lymphatic vessels.
- Demonstrated that Notch receptors and ligands are present both in 231-luc tumors, as well as in their vasculature
- Demonstrated that secretion of a mutant VEGF-C (V_c156s) by 231-luc induces tumor lymphangiogenesis and increases metastasis to lymph nodes

Reportable Outcomes

- Poster presentation for the Era of Hope DOD Breast Cancer Research Program Meeting 2011
- Master of Philosophy, Columbia University, awarded in October 2010

Conclusion

I have demonstrated that Notch is expressed by isolated HDLEC *in vitro*. I have also shown that Notch plays a role in LEC behavior, as inducing Notch signaling affects HDLEC behavior *in vitro*. Induction of Notch signaling also changes the endothelial cell gene profile of HDLEC on the transcriptional level. Though the link between the changes in HDLEC gene profile and changes in *in vitro* activity has not yet been shown, my data suggests that induction of VEGFR-3 by Notch is not the sole factor in Notch's effects on HDLEC *in vitro* behavior. Notch's ability to repress other EC genes such as Prox1, LYVE1, podoplanin, and VEGFR-2 may also play an important role. In future studies, it will be important to study the effects of Notch inhibition in HDLEC. We would expect that Notch inhibition would have the opposite effects of Notch activation.

Additionally, I was able to establish an orthotopic model of human breast cancer that recruits both blood and lymphatic vasculature and metastasizes to lungs and lymph nodes. I have shown that these tumors have Notch-positive blood and lymphatic vasculature. It will be interesting to see how Notch inhibition (using stable cell lines, as well as injection of adenovirus expressing hN1DFc) affects the tumor progression and vasculature in this model.

The goal of this project is to understand the mechanisms involved in a tumor's ability to recruit vasculature, grow, and metastasize. If we observe that hN1DFc treatment is able to inhibit these processes, we may be able to translate these results into the clinical setting. Therefore, this research is highly relevant to breast cancer and potential treatment.